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Structure and Function of the Splice Variants of TMPRSS2-ERG, a Prevalent Genomic Alteration in Prostate Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The most common early genetic defect in prostate cancer (CaP) is the TMPRSS2-ERG gene fusion that results in the expression of the ERG protooncogene. In the context of rearranged genome ERG is transcribed under the control of the androgen inducible TMPRSS2 promoter producing cancer-associated splice variants of ERG. Although, much has been learned in recent years about gene fusions in prostate cancer, the function of TMPRSS2-ERG splice variants is not well understood. However, this information is critical in order to understand ERG functions and its therapeutic targeting in CaP. We have identified two major classes of full-length transcripts of ERG, Type I and Type II splice variants, by screening a cDNA library of TMPRSS2-ERG fusion harboring human prostate tumors. Type I over Type II increased ratios correlated with poor prognostic and clinico-pathologic features of CaP. Moreover, we have found the dominant presence of Type II form in prostate cancer cells. Functional interaction between Type II and Type I splice variants in prostate tumor cells is currently under investigation. Expression of ERG splice variants in human CaP, and the relative ratio of splice variants may provide new strategy in prognosing prostate cancer.

15. SUBJECT TERMS

Prostate cancer, ERG oncogene, Splice variants, TMPRSS2-ERG8, TMPRSS2-ERG3

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Table of Contents

1. <u>INTRODUCTION</u>

We and others have shown that *ETS*-related gene (*ERG*) proto-oncogene is overexpressed in prostate cancer (CaP) transcriptome using microdissected prostate tumor specimens (Ernst *et al.,* 2002; Vanaja *et al.,* 2003; Petrovics *et al.,* 2005). Our studies focusing on comparative quantitative expression of *ERG* in epithelial cells of matched benign and malignant prostate cells from a large patient cohort highlighted that CaP cells harbor frequent overexpression of *ERG* (60-70%) (Petrovics *et al.,* 2005). Among genomic rearrangements described in prostate cancer (CaP), *TMPRSS2-ERG* genomic fusion is the most common mechanism of oncogenic activation (Kumar-Sinha et al., 2008). This gene rearrangement (Tomlins *et al.,* 2005) is due to the fusion of the androgen regulated *TMPRSS2* promoter (Lin *et al.,* 1999; Nelson *et al.,* 1999) to the *ERG* proto-oncogene protein coding sequences (Reddy *et al.,*1987; Rao *et al.,* 1987) in CaP. Detection and characterization of the ERG oncoprotein in CaP and other neoplasms was recently reported by our group (Furusato *et al.,* 2010; Miettinen *et al.,* 2011) and by others (Park *et al.,* 2010; Yaskiv *et al* 2011; Magi-Galluzzi *et al.,* 2011; Minner *et al.,* 2011). Activation of *ERG* has been increasingly recognized as a potentially causal oncogenic alteration (Klezovitch *et al.,* 2008; Tomlins, *et al.,* 2008; Sun *et al.,* 2008; Carver *et al.,* 2009; King *et al.,* 2009; Goldstein *et al.,* 2010). ERG gene is a member of the *ETS* family of transcription factors showing diverse expression patterns in human tissues (Turner and Watson, 2008). *ERG,* similar to other members of the ETS family has been described as a mediator of mitogenic signals, such as, mitogen activator protein kinases (Hart *et al.,* 1995). The multi-exon (17 exons) structure of *ERG* spans about 300 kb, and is transcribed to nine alternative splice variants and isoforms by a combination of alternative transcription initiation, mRNA splicing and transcription termination. The translated products of various *ERG* transcripts can function as oncoproteins with transforming activity (Rao *et al.,* 1987; Sementchenko *et al.,* 1998; Oikawa and Yamada, 2003; Rainis *et al.,* 2005). Thus, due to the functional significance in prostate cancer *ERG* continues to be the subject of intense research investigations. In recent years various *ERG* RNA splice variants have been described, and their relative abundance in CaP cells have been examined (Wang *et al.,* 2006 and 2008, Hu *et al.,* 2008). However, much remains to be defined with respect to *ERG* function in the context of CaP. This knowledge will further our understanding of *ERG* towards its clinical utility including patient stratification, treatment monitoring and therapeutic targeting of CaP.

Towards these goals, we proposed the following specific aims:

#1 Characterization of full length sequences of TMPRSS2-ERG transcripts.

#2 Quantitative evaluations of selected TMPRSS2-ERG variants in CaP specimens and prognostic features.

#3 Defining the functional significance of specific splice variants of the rearranged ERG locus in CaP.

The hypothesis of this proposal is that specific *ERG* splice forms in *TMPRSS2-ERG* fusion configuration are selectively expressed in CaP cells and are functionally relevant in CaP. Since the discovery of *TMPRSS2-ERG* fusions, several fusion transcripts were identified by our and

other laboratories. The expressions of these transcripts were shown to be associated with poor prognosis in previous studies (Wang *et al*., 2006 and 2008; Hu *et al*., 2008). However, careful evaluation of individual full length *TMPRSS2-ERG* transcripts is necessary to understand their biologic functions. Towards defining the functional association, we have generated a cDNA library from tumor specimens of CaP patients to identify and isolate full length *TMPRSS2-ERG* transcripts including the specific splice variants of *ERG* in CaP cells. Characterization of structure and identification of functions of full length *TMPRSS2-ERG* transcripts in CaP will lead to a major advancement in understanding the role of *ERG* in prostate tumor biology.

2. <u>BODY</u>

The progress reported here reflects major advances towards our understanding of key *TMPRSS2-ERG* splice variants expressed in prostate cancer cells

2-1. Summary of Aims and Completed Tasks

Aim #1: Characterization of full length sequences of *TMPRSS2-ERG* transcripts in prostate cancer. Overall aim was to perform innovative analyses of CPDR-CaP- library (at least 1,000,000 pfus) that would facilitate the identification of relatively common TMPRSS2-ERG splice and fusion variants in human CaP.

Completed Tasks

CPDR CaP library was screened with ERG cDNA probe and by TMPRSS2 probe to enrich for TMPRSS2-ERG fusion cDNA clones.

RACE experiments were performed to identify the authentic UTRs from the mRNA pool from CaP patient specimens. Using phage excision strategy, we generated plasmid (cDNA) clones.

DNA sequence of the clones was determined and the sequences were evaluated for open reading frames to identify TMPRSS2-ERG fusion variants and other ERG splice variants.

Aim #2: Quantitative evaluations of selected *TMPRSS2-ERG* variants in prostate cancer specimens and prognostic features.

Towards this objective, we proposed to perform parallel quantitative analyses of up to 6 selected most abundant TMPRSS2-ERG variants in LCM tumor and matching benign epithelial cells from 150 patients representing primary prostate cancer specimens.

We proposed the quantitative expression of the most abundant TMPRSS2-ERG variants with clinico-pathologic parameters including patient age, race, pre-treatment PSA level, Gleason score, CaP family history, tumor stage, surgical margin status, seminal vesicle invasion, PSA recurrence,

bone metastasis, nuclear grade, differentiation, and a follow-up for cancer recurrence by serum PSA after surgery PSA doubling time and prostate cancer associated death.

Furthermore, we proposed to assess the associations of certain ERG transcript variants with genomic deletions associating with poor prognosis by using FISH (fluorescence in situ hybridization).

Completed Tasks

Quantitative RT-PCR analyses of fusion transcripts from 122 evaluable patients have shown the relative abundance of five main ERG transcripts in prostate tumor cells.

Significant correlation was found between increased ratios of type I ERG (ERG1-3) over type II (ERG8 and TEPC1) splice variants and higher Gleason sum and poorly differentiated phenotype.

Towards detecting genomic deletions, FISH assays were performed. TMPRSS2-ERG genomic rearrangements in the sections of primary tumors were examined for TMPRSS2-ERG genomic fusion by dual-color interphase FISH.

Aim #3: Defining the functional significance of specific splice variants of the cancerous ERG locus. In VCaP cell culture model system, we proposed to assess whether *TMPRSS2-ERG* variants (*Type I and Type II*) contribute to the net oncogenic effect of *ERG*. Since the key objectives of Aim 1 and 2 are completed, we are now focused on this aim.

We proposed the evaluation of multiple inhibitory siRNA molecules for specifically targeting specific ERG splice variants expressed in CaP. Experimental objectives were to determine dose response and kinetic measures of inhibition efficacy for each siRNA by measuring the expression of specific TMPRSS2-ERG transcript and protein.

After selecting the most efficient siRNA molecules we planned to evaluate the combined effect of multiple siRNA molecules and measure dose response and kinetic characteristics.

We proposed that individual TMPRSS2-ERG variants (Type I and Type II) would be over expressed using TMPRSS2 promoter driven expression vectors in telomerase immortalized normal prostate cancer cells, such as, RC165 established in CPDR. Also, we proposed the evaluation of cancer biology related features (cell growth, soft agar colony formation, cell invasion and changes in cell cycle of cell) of the cells in response to knocking down or heterologous expression of specific TMPRSS2-ERG transcripts.

We proposed to test predicted protein products from Type I and Type II TMPRSS2-ERG transcripts for their ability and specificity to bind to target sequences in gel-shift experiments. We proposed to evaluate the regulatory efficiency of Type I and Type II products by using different known promoters (TGFβ type II receptor, MMP3 and collagen) by using luciferase assay systems.

Completed Tasks

Inhibitory siRNA molecules were designed and prepared to specifically target the abundant ERG8 (Type II) and ERG1-3 (Type I) transcripts. The dose and kinetic assessment of siRNA molecules were performed for the inhibition of Type I and Type II transcripts.

Nuclear localization of transiently expressed in HEK293 cells were evaluated in the presence of TMPRSS2-ERG3 and TMPRSS2-ERG8 coded protein products of ERG.

The regulatory efficiency of Type I and Type II products were tested by using different ERG targeted promoters in luciferase assay systems.

2-2. Progress Highlights for the Reporting Period

Identification of an *ERG8*-specific siRNA for the inhibition of the predominant *Type II* splice variant of ERG. We have designed and evaluated specific siRNA molecules for knocking down the *Type II* splice variant *ERG8* in HEK293 cells transiently transfected with *TMPRSS2-ERG8* and *TMPRSS2-ERG3* expression vectors. The experiment revealed that inhibition of *ERG8* by siRNA significantly reduced the expression of ERG8 protein levels in HEK293 cells. Towards the goals of this specific aim to generate siRNAs that specifically inhibit Type II variants we have generated a siRNA for the knockdown of ERG8 without interfering with the expression of protein products of Type I transcripts.
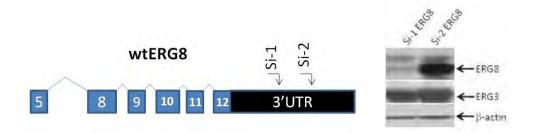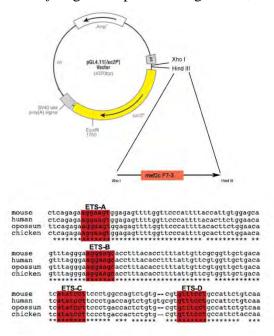

Figure 1. *Inhibition of ERG8 protein in response to ERG8 selective siRNA. We have designed siRNA molecules for targeting specific ERG splice variants and siRNAs were assayed for inhibiting ERG8. HEK293 cells were co-trasfected with TMPRSS2-ERG8 and TMPRSS2-ERG3 expressing plasmid vectors and siRNA molecules targeting ERG8. Selective inhibition of the ERG8 protein in immunoblot assay (right panel) demonstrates the identification of a siRNA specific for ERG8. The ERG3 protein, encoded by Type I was unaffected by ERG8 siRNA.*

Evaluation of the transcription regulatory function of Type I and Type II products by using luciferase assay systems. The Myocyte Enhancer Factor 2 (MEF2) family of transcription factors are expressed in multiple cell types and are important regulators of development and differentiation (DeVal *et.al*, 2004; Wei *et al*., 2010). The enhancer from the mouse *mef2c* gene is a well characterized enhancer that has been shown to be active in the vascular endothelium during embryogenesis and in adulthood where endogenous ERG protein is highly expressed (Mohamed *et al.,* 2010; Furusato *et al*., 2010). Therefore, to evaluate the transcriptional regulatory function of ERG splice variants, we have utilized the *mef2c* vascular endothelial enhancer sequence, which harbors a cluster of four conserved elements for ETS factors, including ERG. Although we had
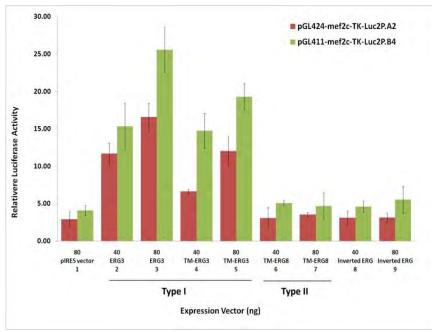
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originally intended to use other regulatory sequences (*TGF-beta*, *MMP3* and *collagen*), on the basis studies of *mef2c* gene expression regulation, we anticipated a strong reporter read out using *mef2c*

enhancer based luciferase reporter construct when transcriptional regulatory activities of *ERG* splice variants were assayed. The *mef2c* enhancer was cloned into the pGL4.24[*luc2P*/minP] vector that carries a minimal promoter upstream of the luciferase reporter gene *luc2P*, which is designed for high expression, reduced anomalous transcription and sensitivity to activation and repression.

Figure 2. Design of a Luciferase reporter vector by using the mouse mef2c vascular endothelial enhancer for evaluation of transcriptional regulatory function of ERG variants.

The pGL4.24-*mef2c*-[*luc2P*/minP] reporter construct was co-transfected with 40 ng and 80 ng of expression vectors encoding Type I and Type II ERG proteins in HEK293 cells. Both the full length (ERG3) and the N-terminus truncated proteins (products of TMPRSS2-ERG fusion) expressed activated the transcription from the *mef2c* driven luciferase reporter construct. However, activation by the N-terminus truncated ERG3 protein is 10-20% lower than activation by the full length protein. Consistent with the absence of nuclear localization signal in Type II encoded ERG8,

this ERG variant did not alter the basal promoter activity of the reporter construct.

Figure 3. Type I transcript encoded ERG protein (ERG3 and the N-terminus truncated ERG3 protein, TM-ERG3) activates the transcription of luciferase reporter through the mef2c promoter in a dose dependent manner. Consistent with the absence of nuclear localization signal in Type II encoded ERG, ERG8 does not alter the basal promoter activity of the reporter construct.

In an experiment addressing the interaction of *Type I* and *Type II* splice variants towards Specific Aim #3 we co-transfected *TMPRSS2-ERG3* and increasing concentrations of *TMPRSS2-ERG8* expression vectors in HEK293 cells. We found a dose dependent inhibition of ERG3-mediated activation of reporter in response to increasing doses of *ERG8*. This observation support the central hypothesis of our proposal that the *Type II* splice form may interfere with *Type I* function. However, further experiments are needed to follow up on these preliminary observations.
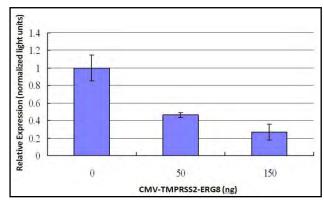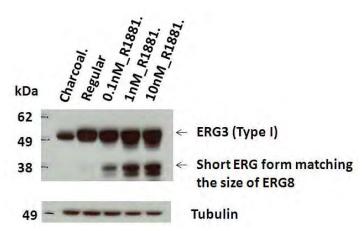

Figure 4. Dose dependent abrogation of ERG3 (Type I)-mediated transcriptional activation by increasing concentrations of ERG8 (Type II). HEK293 cells were co-transfected with TMPRSS2-ERG3 and 0, 50 and 150 ng of TMPRSS2-ERG8 expression vector. Luciferase activity was measured in light units and normalized to the control (0 ng of TMPRSS2-ERG8 vector).

Expression of ERG proteins in cell lines The highly-specific anti-ERG monoclonal antibody (CPDR ERG-MAb) developed in our laboratory recognizes ERG proteins encoded by both type I and II splice variants, this antibody has provided a unique opportunity for the evaluation of all ERG protein forms in prostate and other cancers (Furusato *et al*., 2010; Miettinen *et al*., 2011). In this reporting period we have further evaluated ERG protein translated from Type I and Type II splice variants in various cancer cell lines. We have examined the androgen inducible expression of ERG

proteins in TMPRSS2-ERG harboring VCaP cell line in response to increasing doses of the synthetic androgen hormone R1881. As expected, we observed the dose dependent increase in ERG3 protein expression. Under these experimental conditions, we also observed the increased expression of a protein product with molecular weight similar to the protein product of *Type II* transcript, ERG8. This observation further highlights the need for the evaluations of ERG 8 protein that has not been studies before.

Figure 5. Androgen dose dependent expression of ERG3 protein, a product of Type I splice variant and the expression of a 38 kDa ERG protein matching the expected size of ERG8 (Type II).

Similarly, we have observed ERG3 and shorter protein products in cell lines derived from diverse cancers: acute myeloid leukemia, KG-1; colon cancer, COLO 320; acute T lymphoblast leukemia, MOLT4; and prostate cancer, VCaP cells. In addition to the expression of Type I splice variant product we consistently detected shorter protein products. The relation to the Type II splice variant ERG8 to the observed protein products will be further examined.

Figure 6. Detection of ERG protein in prostate and non-prostate cancer cell lines CPDR ERG-MAb. ERG protein expression was analyzed by using cell lysates from cell lines derived from diverse cancers: Jurkat (acute T cell leukemia); MCF7 (breast cancer), KG1 (acute myelogenous leukemia), COLO320 (colon carcinoma); MOLT-4(acute lymphoblastic leukemia); VC-NT (VCaP prostate cancer cells treated with non target siRNA); VC-si-1(VCaP prostate cancer cells treated with ERG specific siRNA). LNCaP (prostate cancer cell line, Jurkat, MCF7 and LNCaP cells were used as negative controls.

Temporal/spatial expression of ERG proteins in developing mouse. As discussed above the highly-specific anti-ERG monoclonal antibody (CPDR ERG-MAb) developed in our laboratory recognizes ERG proteins encoded by both *type I* and *II* splice variants. Using this antibody, we have established Erg protein in developing and adult mouse tissues. Most striking of these observations was highly selective and abundant expression of erg protein in endothelial cells of mouse tissues. We for the first time clarified that endogenous ERG was not expressed in normal mouse prostate epithelium (Mohamed *et al*., 2010). Similar findings were noted for human prostate (Furusato *et al.,* 2010). These observations underscored cancer specific aberrant expression and function of ERG in prostate epithelium.
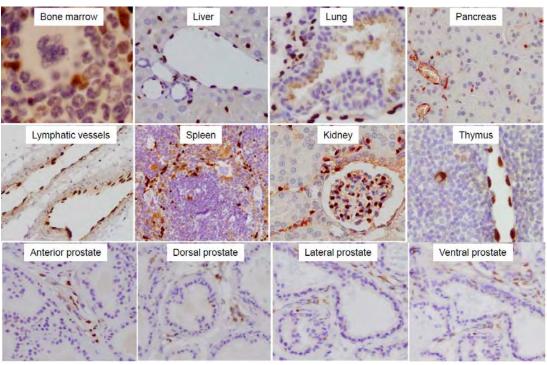

Figure 7. Widespread immunolocalization of Erg proteins was observed in endothelial cells and restricted expression in precartilage and hematopoietic tissues. Erg is not expressed in any epithelial tissue including prostate epithelium(lower panels), or in infiltrating lymphocytes that are occasionally seen in the prostate environment, a common site of tumors with ERG rearrangements and unscheduled ERG expression.

To assess the Type II splice variant, ERG8, we have developed a rabbit polyclonal anti-ERG8 antibody. Initial characterization of the anti-ERG8 antibody confirmed the specificity of the antibody in detecting ERG8 protein by Western blot assays. This antibody will be further evaluated.

3. <u>KEY RESEARCH ACCOMPLISHMENTS</u>

- Transcriptional regulatory activities of *ERG* splice variants were assayed by using murine *mef2c* promoter-luciferase reporter construct. Type I variant encoded ERG3 protein activates the transcription of luciferase reporter through the *mef2c* enhancer. In contrast, type II encoded ERG8 does not alter the basal promoter activity.

- We have delineated the temporal and spatial expression profile of ERG oncoprotein during mouse embryo development by using the highly specific CPDR ERG-MAb developed by our laboratory.

- Erg is not expressed in any normal epithelial tissue including prostate epithelium, or in infiltrating lymphocytes that are occasionally seen in the prostate environment, a common site of tumors with ERG rearrangements and unscheduled ERG expression.

- We have developed ERG 8 selective siRNAs and antibodies which will facilitate ERG8 expression and functions.

To complete Specific Aim #3, the following experiments are in progress and will be performed in the next reporting period. While we have successfully accomplished several key objectives of the grant proposal, with the unscheduled early departure of the post-doctoral fellow primarily assigned to this project within the approved extension we will complete 1) the molecular and biological assessment of delivering inhibitory molecules with targeting prognostically relevant fusion transcripts in *TMPRSS2-ERG* harboring VCaP cells; 2) we will assess changes in cell growth, invasion and differentiation characteristics of normal prostate cells in response to the ectopic expression of *TMPRSS2-ERG* splice variants; 3) predicted protein product of *TMPRSS2-ERG* splice variants will be tested for their ability and specificity to regulate different known promoters and for their sub-cellular localization.

4. <u>REPORTABLE OUTCOMES</u>

4-1. Publication

Mohamed AA, Tan SH, Mikhalkevich N, Ponniah S, Vasioukhin V, Bieberich CJ, Sesterhenn IA, Dobi A, Srivastava S, Sreenath TL. Ets family protein, erg expression in developing and adult mouse tissues by a highly specific monoclonal antibody. J Cancer. 2010 Oct 25;1:197-208.

4-2. Patent Application

ERG Monoclonal Antibodies. PCT/US2010/032714 , WO/2010/126972, published on November 4, 2010.

4-3. Press Release

None

4-4. Poster presentations

DoD-PCRP IMPACT Meeting 2011

Structure and Function of the Splice Variants of TMPRSS2-ERG, Prevalent Genomic Alteration in Prostate Cancer.
Taduru Sreenath, Albert Dobi, Ying Hu, Shyh-Han Tan, Yongmei Chen, Chen Sun, Isabell A. Sesterhenn, David G. McLeod, Gyorgy Petrovics and Shiv Srivastava

Sub-cellular Localization and Transcriptional Activity of ERG Protein Encoded by the Common *TMPRSS2-ERG* Splice Variants Expressed in Prostate Cancer.
Zainab Afzal, Tseday Zewdu Tegegn, Ahmed Mohamed, Taduru Sreenath, Deepak Kumar, Shiv Srivastava and Shyh-Han Tan

AACR Annual Meeting, 2011

ETS-Related Gene (ERG) Expression in Developing and Adult Mouse Tissues
Ahmed A. Mohamed, Shyh-Han Tan, Natallia Mikhalkevich, Sathibalan Ponniah, Valeri Vasioukhin, Charles J. Bieberich, Isabell A. Sesterhenn, Albert Dobi, Shiv Srivastava and Taduru L. Sreenath

5. <u>CONCLUSIONS</u>

- Identification of *TMPRSS2-ERG*, *Type I*, which encodes full-length ERG protein consisting SAM and ETS domains (ERG1, ERG2, ERG3), and *Type II,* encoding ERG proteins lacking the ETS domain (ERG8 and a new variant, TEPC)

- Quantitative RT-PCR analyses of fusion transcripts from 122 patients showing relative abundance of ERG *Type I* and *Type II* splice variants

- Increased ratio of *Type I* over *Type II* variants showing correlation with poorly differentiated pathology /high Gleason score and outcome

- Transient expression in HEK293 cells revealed the presence of *TMPRSS2-ERG3* encoded protein (Type I) in the nucleus and *TMPRSS2-ERG8* encoded protein (*Type II*) in the cytoplasm

- Successful developed of a siRNA for the inhibition of ERG8 *Type II* splice variant.

- Demonstration that ERG3 a product of *Type I* splice variant, transcriptionally activates gene expression through ETS-regulated enhancer and co-transfection of the ERG8 expressing *Type II* splice variant abrogates the transcriptional activator function of ERG3 (*Type I*).

- Generation of a novel rabbit polyclonal antibody for detecting ERG8 protein in human prostate cancer specimens and currently evaluating this antibody.

- Delineation of the expression of Erg protein during development. Demonstrated normal expression of ERG protein in endothelial cells, and transient expression in precartilage and hematopoietic tissues. Revealed that ERG is not expressed in normal epithelial tissues including normal prostate epithelium.

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7. <u>**APPENDICES AND SUPPORTING DATA**</u>

Research Paper

Ets Family Protein, Erg Expression in Developing and Adult Mouse Tissues by a Highly Specific Monoclonal Antibody

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Abstract

Oncogenic activation of the ETS Related Gene (*ERG*) in humans was originally identified in subsets of Ewing sarcomas, myeloid leukemias and, recently, in the majority of prostate cancers. Expression of human ERG protein and consequently its functions in normal and disease states needs to be better understood in light of its suggested role in cell differentiation and proliferation. Here, we analyzed temporal and spatial expression of the Erg (mouse protein) by immunohistochemical analysis during mouse embryonic and adult organogenesis using a highly specific ERG monoclonal antibody (ERG MAb). This study establishes widespread immunolocalization of Erg protein in endothelial cells and restricted expression in precartilage and hematopoietic tissues. Intriguingly, Erg is not expressed in any epithelial tissue including prostate epithelium, or in infiltrating lymphocytes that are occasionally seen in the prostate environment, a common site of tumors with *ERG* rearrangements and unscheduled *ERG* expression. These findings will further aid in investigations of Erg functions in normal and disease conditions.

Key words: Ets Related Gene, ERG, Expression, ERG MAb, Mouse, Development.

Introduction

Chromosomal translocations leading to gene fusions have been well characterized in variety of malignancies [1]. Gene fusions often result in chimeric proteins with aberrant functions and/or ectopic expression. Frequent elevated expression of the *ETS* related genes (*ERG*) due to chromosomal rearrangements resulting into the fusion between androgen regulated promoters (predominatly*TMPRSS2*) and protein coding sequence of *ETS* transcription factors

has been established in prostate cancer [2, 3]. *ETS* (Erythroblast Transformation Specific family of transcription factors) genes are a large family with at least thirty members that function as transcription factors [4]. All *ETS* transcription factors share a highly conserved DNA binding domain, the ETS domain [5] and at least *Ets1, Erg, Fli1* and *Etv2* are expressed in embryonic endothelial cells of mouse [6, 7]. *ERG* is well conserved in evolution and its expression and poten-

tial functions have been studied in xenopus, zebrafish, mouse and humans [8-12]. The results from these studies suggest an emerging role for *ERG* in the transcriptional regulation of endothelial specific genes [13-16] and in definitive hematopoiesis [17, 18]. Both hematopoietic and endothelial cells are of mesodermal origin and are derived from the hemangioblast, a common precursor, suggesting a shared developmental pathway [19]. Knock-down of *Erg* is associated with a significant reduction in the formation of vascular structures and the number of endothelial cells [20] and with apoptosis [21]. These studies indicate that Erg may have important implications in vascular development during mouse embryogenesis. Although *Erg* does not appear to be required for hematopoiesis during embryonic stem cell differentiation, it may play a role in endothelial cell differentiation [20]. Hematopoietic stem cells give rise to both T- and B-lymphocytes in embryogenesis and throughout adult life. Although mature T-lymphocytes do not express *Erg*, expression is detected transiently during T-lineage specification and is silenced after their commitment [22]. During B-cell development in the mouse, *Erg* expression was detected in early pre-B cells, pre-B and in mature B cells [23]. In developing mouse, *Erg* mRNA is expressed in mesodermal tissues such as endothelial cells, mesenchymal condensations during precartilaginous depositions, and in urogenital regions [11]. All of the expression studies were carried out by using RT-PCR or in situ hybridization. However, the protein expression and its cellular distributions could not be performed due to a lack of an Erg-specific antibody.

The goal of this study was to establish the expression pattern of Erg protein in developing and adult mouse tissues by using an ERG-specific antibody. These data would serve as a basis to understand the function of Erg during normal development in many organs and pathological conditions, such as its cancer-specific expression in prostatic adenocarcinoma. Although several antibodies for detecting human ERG protein and mouse Erg protein have been described, due to high degree of homology among *ET*S family members, in particular its closest homologue *Fli-1*, antibody cross reactivity has become a major concern in detection of the ERG protein. Recently, we have generated and characterized an ERG-specific mouse monoclonal antibody that showed high specificity towards ERG protein that does not cross react with FLI-1 protein [24]. In the present study, we examined the detailed expression of the Erg protein during prenatal and adult mouse organogenesis.

Results and discussion

Evaluation of ERG Monoclonal Antibodies for the Specificity of ERG Protein Detection: We evaluated the specificity of three recently available ERG monoclonal antibodies including the ERG MAb that we recently reported [24]. As noted previously, the ERG MAb detected ERG protein products in MOLT4, KG1, COLO 320 and VCaP tumor cell lines, whereas LNCaP, MCF7 and Jurkat cell lines were negative for ERG. The ERG MAb did not show cross reactivity to FLI-1 in LNCaP cells infected with a FLI-1 adenovirus expression vector (Fig 1A). Under similar assay conditions, rabbit monoclonal antibodies to ERG (EPR 3864 and EPR 3863) obtained from Epitomics (Burlingame, CA) detected FLI-1 in LNCaP cells infected with a FLI-1 adenovirus expression vector (Fig 1B, 1C). In addition, a rabbit monoclonal ERG antibody EPR 3864 detected a protein in Jurkat cell line (acute T cell leukemia) that was not recognized by either the rabbit monoclonal antibody EPR 3863 or our ERG MAb suggesting potential cross reactivity to other ETS related proteins by EPR 3864. Interestingly, other monoclonal ERG antibody EPR 3863 recognized FLI-1 in LNCaP cells infected with a FLI-1 adenovirus expression vector. Taken together, the results obtained from immunoblot analyses suggest that the ERG MAb we developed is highly specific for ERG protein detection and was further assessed in other immunoassays.

To determine the efficiency of ERG MAb antibody in a prostate tumor model, we analyzed ERG expression in ERG-positive VCaP and ERG-negative LNCaP prostate cancer cell tumor xenografts in SCID mice. The ERG MAb antibody detected ERG protein in VCaP xenografts, staining primarily the nuclei with some cytoplasmic reactivity (Fig 2A). As expected, ERG negative LNCaP xenografts did not show ERG expression. The endothelial cells lining the blood vessels and capillaries showed positive reactivity to ERG MAb in tumors as well as normal adjacent tissue (Fig 2B). Further, we evaluated the ERG MAb for the detection of ERG protein the prostates of ERG-transgenic mice [25]. Transgenic ERG expression was detected in the prostate luminal epithelial cells of ERG-transgenic mice (Fig 2C). In addition, endogenous Erg protein was detected only in the endothelial cells of blood vessels and capillaries (Fig 2D). Infiltrating lymphocytes did not show immune-reactivity to ERG MAb (Fig 2 E, G). Consistent with a recent report [26], rabbit monoclonal antibodies EPR 3864 showed a strong staining of lymphocytic infiltration in prostate gland (Fig 2F, H). Interestingly, both the rabbit monoclonal ERG antibodies (EPR 3864, EPR

3863) and FLI-1 antibodies have shown reactivity to infiltrating lymphocytes (Fig 2I, J). It is interesting to note that ERG is turned on at transition stage (between precursor and pro-T stage 1) of T-cell differentiation and does not persist stably to define T-cell identity, and is shut off after T cell lineage commitment [22, 27, 28]. In this study, we have not characterized the transient expression of Erg during T-cell development and differentiation.

To study the utility of the ERG MAb to detect the expression of ERG in cells using flow cytometry, seven human cancer cell lines, VCaP and LNCaP (prostate cancer), T2 and Jurkat (T lymphoblastoid cells), KG-1 and KG-1a (myeloblastic) were analyzed. All cell lines were permeabilized by standard cell intracellular staining methods by using detergent followed by incubation with the ERG MAb and secondary fluorochrome-conjugated antibodies. As shown in Fig 3C and D, expression of ERG was clearly detectable in the TMPRSS2-ERG fusion-harboring VCaP cells and was not observed in the TMPRSS2-ERG negative LNCaP cells (Fig 3A, 3B). In the case of the hematopoietic/lymphoblastoid cancer cells of the T lymphocyte lineage, ERG expression was detected neither in T2 (Fig 3I, 3J) nor in Jurkat cells (Fig 3K, 3L). With the hematopoietic cell lines of myeloid lineage, KG-1 and KG-1a, the expression of ERG was clearly detectable in both cell lines (Fig 3E, 3F and Fig 3G, 3H). Interestingly, the KG-1a cells, which are considered to be less mature or differentiated than the KG-1 cells, expressed much higher amounts of the ERG protein. Taken together, the consistent results of Western blot, IHC and FACS assays established the specificity of the ERG MAb in detecting ERG protein in different assay platforms and biological specimen contexts.
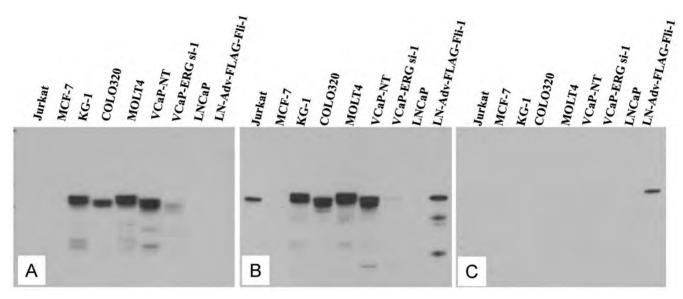

Figure 1: Expression of human ERG protein in cancer–derived cell lines: ERG protein expression was analyzed by using total cell lysates from acute T cell leukemia (Jurkat), breast cancer cell line (MCF7) acute myelogenous leukemia cell line (KG1), colon carcinoma cells (COLO320), acute lymphoblastic leukemia (MOLT-4), ERG expressing prostate cancer cell line (VCaP) treated with non target siRNA (VCaP-NT), prostate cancer cell line (VCaP) treated with ERG specific siRNA (VCaPERG-si-1), prostate cancer cell line LNCaP, prostate cancer cell line LNCaP transduced with adenoviral FLI-1 expression vector. Extracted proteins were processed for immunoblot assay by using (A) mouse monoclonal ERG antibodies ERG MAb, (B) rabbit monoclonal anti-ERG antibodies Epitomics EPR 3864 Cat.No 2805-1, (C). Rabbit monoclonal anti-ERG antibodies Epitomics EPR 3863 Cat.No 2849-1. Note the lack of immunoreactivity to the protein extracts from Jurkat, LNCaP and LNCaP transduced with adenoviral Fli-1 expression vector with ERG MAb in panel A, and reactivity with other antibodies in panel B and C.

Figure 2: Expression of ERG protein in FFPE tissue: ERG protein expression in (A) VCaP and (B) LNCaP xenograft tumors obtained from SCID mice. Strong expression of ERG in VCaP cells (black arrow). ERG staining is seen only in the endothelial cells (arrow) of LNCaP tumors but not in the epithelial cells. Expression of ERG protein is detectable in the ARR2PBΔERG transgenic mouse prostates (C) compared to wild-type littermate control (D). Infiltrating lymphocytes are occasionally seen in the prostate glands and show no reactivity to ERG MAb (E, G). However, strong staining is seen in the infiltrating lymphocytes with Epitomics EPR 3864 (F, H), EPR 3863 (I) and FLI1 antibody (J).
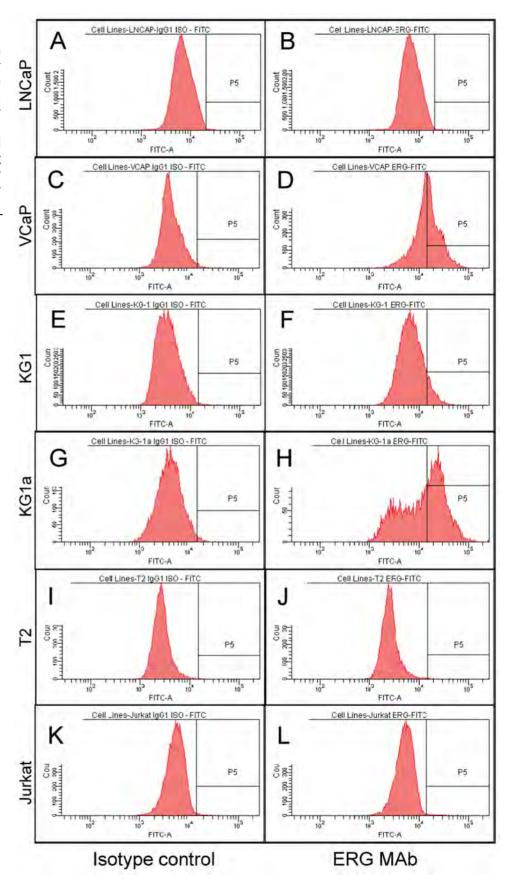
Figure 3. ERG expression in cancer cell lines. Intracellular staining was carried out in permeabilized cells with an IgG1 isotype control antibody (A, C, E, G, I and K) or for ERG with ERG MAb (B, D, F, H, J and L) and detected by using FITC-conjugated secondary antibody and flow cytometry analysis.

Isotype control   ERG MAb

Erg protein expression in prenatal mouse development: During E9.5d, strong expression of Erg protein was detected by the ERG MAb mainly in the endothelial cells of blood vessels around the neural tube (Fig 4A, 4B). Additionally, expression was also prominent in the cells that line the amnion (Fig 4A). At this stage, during heart development, the ventricle begins trabeculation to demarcate this region from the primitive heart. Expression of Erg was observed in endothelial cells that line the trabeculated regions of the ventricle (Fig 4C). Endothelial cells present in the inter-somitic capillaries show strong expression of Erg (Fig 4D). Similar endothelial-specific expression was found in the dorsal aorta and around the neural tube (Fig 4E). The distribution observed with ERG MAb antibody is consistent with earlier reported studies of *Erg* mRNA expression [11, 20]. Similar to earlier stages of development, at E12.5d, Erg expression was endothelial cell-specific in the majority of the tissues (Fig 5). In addition to endothelial expression, Erg expression was detected in the precartilage/ cartilage primordium of the nasal septum, neural arch and rib (Fig 5A, 5B, 5C). Mesenchymal condensations are required at this stage to initiate the paving cartilage path for both transient and permanent cartilage. The transient cartilage will undergo ossification to form bone. Interestingly, Erg expression was observed only in the precartilage primordium suggesting that Erg may have critical role in the differentiation of cartilage. Heart development at this stage exhibited extensive trabeculation of the ventricle and showed clear lining of endothelial cells with positive Erg staining along the trabeculated endocardium (Fig 5B). Lungs at this stage were not yet divided into lobes and the stroma with enriched capillaries exhibited strong expression of Erg in developing lung (Fig 5D). Epithelial cells of segmental bronchus did not show Erg expression (Fig 5D). Kidney at this stage starts subdividing into cortical and medullary regions. Expression was detected only in the blood vessels and capillaries uniformly throughout the kidney and not in the kidney cortex or medulla (Fig 5E).
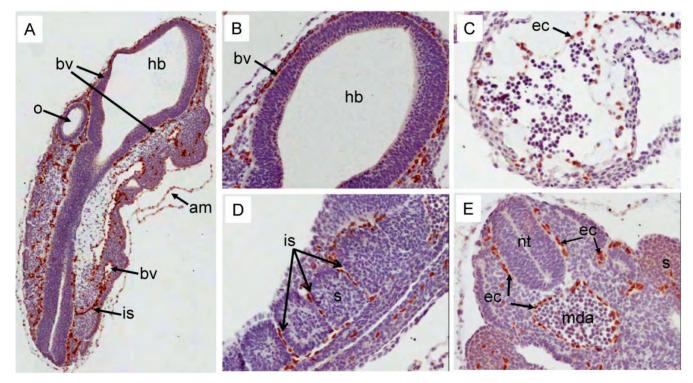

Figure 4. Expression pattern of Erg protein during mouse embryogenesis (E9.5d): Embryonic 9.5d mouse showing the expression of Erg protein by immunohistochemistry with ERG MAb. (A) Coronal section of an E9.5 embryo showing a specific staining in blood vessels (bv) , inter-somitic vessels (is) and in the amnion (am). (B) Higher magnification of hind brain. Expression is not seen in the hind brain (hb), neural tube (nt) and optic vesicle (o). (C). Higher magnification of ventricle (vt) region of the heart showing strong signal in the endothelial cells (ec)along the trabeculated endochordium (D) Hihger magnification of somites in the caudal region showing Erg expression in the inter somatic blood vessels (sv) . (E). Tail region of the embryo showing neural tube (nt) midline dorsal aorta (mda). Erg expression was detectable only in the endothelial cells of dorsal aorta. Somites (s).
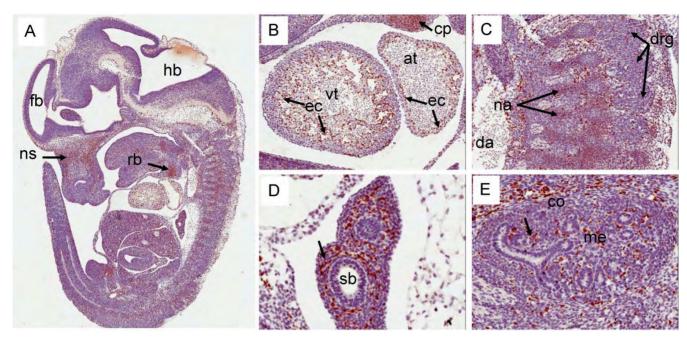
Figure 5. Expression pattern of Erg protein during mouse embryogenesis: (E12.5). (A) Sagittal section of an E12.5d embryo showing a specific staining in cartillage primordium (cp) of the nasal septum (ns), and the mid shaft region of the rib (rb). (B) Higher magnification of ventricle (vt) region of the heart showing strong expression in the endothelial cells (ec) along the trabeculated endocordium. (C) Erg protein was detectable in the precartillage condensations in the neural arch (na). (D) Higher magnification of developing lungs (not yet divided into lobes) show lack of expression in the epithelial cells of segmental bronchus (sb). Surrounding stroma with enrihed capillaires exhibit strong staining. (E). Expression is seen only in endothelial cells of the blood vessels and capillaries uniform throughout the kidney.

Erg expression in E14.5d was found mostly in the endothelial cells of variety of tissues (Fig 6). In developing liver, about 1-2% of liver cells exhibited reactivity with ERG MAb antibody and megakaryocytes did not show Erg expression. Expression in the cartilage appeared to be reduced significantly compared to E12.5d in the rib, nasal septum and vertebrae. We found significant differences between ERG MAb and rabbit monoclonal ERG antibodies EPR 3864 megakaryocytic immunostaining. No significant changes in the Erg expression patterns were observed in the later stages of mouse development. As the overexpression of ERG in the prostate is implicated in the oncogenic process, we examined the normal expression of Erg protein in developing prostate glands. In the mouse, the prostatic buds first emerge at the rostral end of the urogenital sinus at approximately 17.5 days of gestation and subsequently, the prostatic epithelial buds undergo extensive ductal outgrowth and branching morphogenesis into the surrounding mesenchyme during the first three weeks of postnatal development. Interestingly, Erg staining was observed only in the mesenchymal compartment and restricted to capillary endothelial cells (Fig 7) suggesting that Erg may not be critical in normal prostate development or differentiation.

In liver of E17.5d mice, the expression of Erg is restricted to very few cells such as endothelial and other non-parenchymal cells which later differentiate into Kupffer cells and hepatic stellate cells. It is not clear at this stage which type of cells in addition to endothelial cells express Erg protein. Similar to E14.5d liver megakaryocytes, ERG MAb did not show reactivity with E17.5d hepatic megakaryocytes (Fig 8A). However, rabbit monoclonal ERG antibodies EPR 3864 showed a strong staining in megakaryocytes, in addition to endothelial and other cell types (Fig 8B).

Expression of Erg protein in adult mice: We extended our study to adult mouse tissues to analyze the expression of Erg protein (Fig 9). Similar to embryonic tissues, the expression of Erg was observed in the endothelial cells of the adrenal gland, cartilaginous component of bone, heart muscle, kidney, liver, lung, spleen urinary bladder. Erg expression was also evident in the lymphatic endothelial cells in adult mouse. As expected, expression was undetectable in the prostate epithelium. Detailed examination of these tissues has revealed that the expression was mostly restricted to hematopoietic and endothelial compartments. In liver, the expression appeared to be in the Kupffer cells. In bone marrow, ERG MAb did not show reactivity with megakaryocytes. Interestingly,

eosinophils, with a characteristic ring shaped or segmented/multilobed nuclei stained the nuclei with ERG MAb. Eosinophils are derived from hematopoietic stem cells initially committed to the myeloid line and then to the basophil-eosinophil granulocyte lineage. The presence of Erg in these cells suggest its potential function in either differentiation or maintenance of differentiation state. During development, Fli1 is preferentially expressed in hematopoietic cells, endothelial cells and in the mesenchyme which is mainly derived from neural crest cells [29]. Similar to earlier observations [11] we also observe the co-expression of the *Erg* and *Fli1* genes in both endothelial and mesodermal tissues, including urogenital tract and precartilaginous areas. Similar to Erg, Fli1 expression also appeared transient during mouse embryogenesis. This result is consistent with subtle temporal regulation. Whether this spatiotemporal overlap determines redundant functions or not remains to be determined. Due to specificity and clarity of Erg detection in developing and adult mouse tissues, these findings will be valuable in further assessing in vivo functions of Erg in normal and malignant tissues.
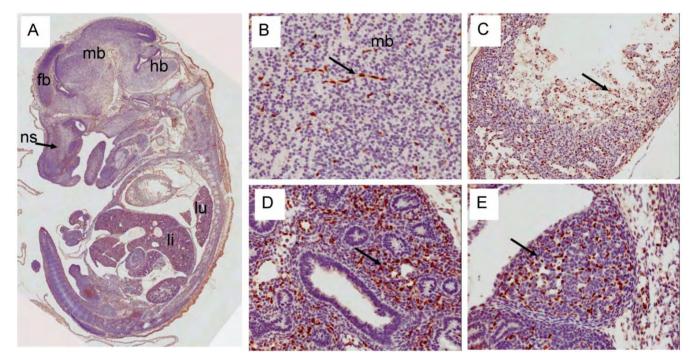

Figure 6. Expression pattern of Erg protein during mouse embryogenesis (E14.5): (A) Sagittal section of an E14.5 embryo showing a specific staining in cartillage primordium (cp) of the nasal septum (ns) similar to E12.5d. Higher magnification of midbrain showing capillaries staining of Erg. (C) Higher magnification of ventricle (vt) region of the heart showing strong ractivity in the endothelial cells (ec) along the trabeculated endocordium. (D) Higher magnification of developing lungs shows lack of expression in the epithelial cells of segmental bronchus (sb). Stroma with enrihed capillaires exhibit strong staining. (E). Higher magnification of adrenal gland with endothelial cell specific Erg staining.
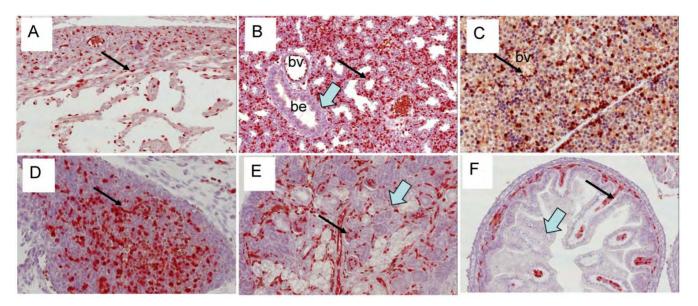
Figure 7. Expression pattern of Erg protein during mouse embryogenesis(E17.5): (A) Ventricle showing trebeculated endocordium with endothelial specific staining of Erg. (B). High magnification of lung showing capillaries and blood vessel specific Erg reactivity. Note the lack of staining in the lung epithelial. (C) High magnification of liver showing a specific staining is non-hepatocytes in addition to endothelial cells. (D) High magnification of adrenal gland showing random Erg in both cortex and medullary region consistent with fenestrated vessels. Similarly, kidney (E) and intestine (F) show endothelial specific staining, Open arrows show the lack of expression in the bronchial epithelial cells (B), kidney tubules (E) and intestinal epithelial cells in the cripte (F). Blood vessel (bv), bronchial epithelium (be).
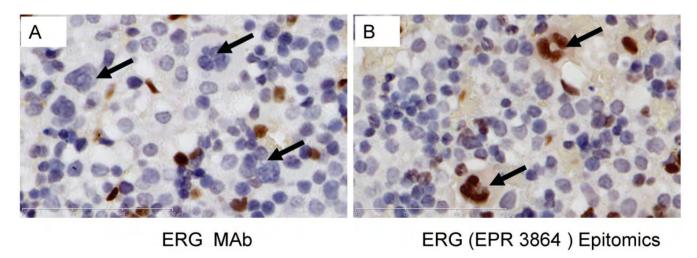

ERG MAb   ERG (EPR 3864) Epitomics

Figure 8. Expression of Erg protein in developing liver (E17.5). (A) Detection of Erg protein in endothelial and non-paranchymal cells by ERG MAb. The arrows point to megakaryocytes that lack the expression of Erg protein. (B). Erg staining with rabbit monoclonal ERG antibodies Epitomics EPR 3864 show endothelial and non-paranchymal cells staining. Megakaryocytes know to have expression of Fli1 also are detected with EPR 3864. Similar megakaryocyte specific expression is also observed with EPR 3863 and FLI1 antibodies (data not shown).
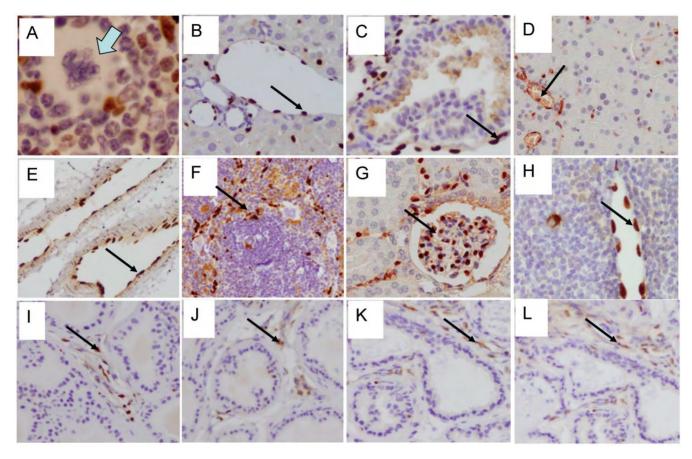
Figure 9. Expression of Erg protein in adult tissues: (A) Bone marrow (B) Liver (C) Lung (D) Pancreas (E) Lymphatic vessels (F) Spleen (G) Kidney (Glomeruli) (H) Thymus (I) Anterior prostate (J) Dorsal prostate (K) Lateral prostate (L) Ventral prostate. Expression is seen mainly in the endothelial cells in blood vessels and capillaries (arrows). In bone marrow, megakaryocyte show lack of Erg expression (open arrow).

Materials and Methods

Antibodies: Recently, we have reported the generation and characterization of mouse monoclonal antibodies to ERG, showing higher specificity [24]. In this study, we have used mouse monoclonal ERG antibodies along with the other commercially available ERG antibodies, a rabbit anti-ERG monoclonal antibody clone EPR 3864 (Cat No. 2805-1) [26] and clone EPR 3863 (Cat No. 2849-1) obtained from Epitomics, Burlingame, CA and FLI-1 antibodies from Dr. Denis Watson, University of South Carolina, Charleston, SC. Anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA) sheep anti-mouse IgG-HRP (NXA931, GE Health Care, Buckinghamshire, UK) donkey anti-rabbit IgG-HRP (NA934, GE Health Care, Buckinghamshire, UK)

Western blot assays. Cells (Jurkat, MCF7, KG1, Molt4, VCaP, LNCaP) were lysed in Mammalian Protein Extraction Reagent (M-PER) (Pierce, Rockford, IL) containing protease inhibitor cocktail and phosphatase inhibitor cocktails I & II (Sigma, St Louis,

MO). Cell lysates equivalent to 50 µg of protein were separated on 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to PVDF membrane (Invitrogen, Carlsbad, CA). Membranes were incubated with primary antibodies: Anti-ERG mouse monoclonal antibody (ERG MAb)[24] (1:500 dilution), rabbit monoclonal ERG (EPR 3864 and EPR 3863) antibodies (1:500 dilution) anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for overnight. Membranes were washed three times for 5 minutes each at room temperature followed by treatment with secondary antibodies: sheep anti-mouse IgG-HRP or donkey anti-rabbit IgG-HRP at 24°C for 1 hour. Finally membranes were washed three times and bands were visualized with ECL Western blot detection reagent (GE Health Care, Buckinghamshire, UK).

Immunofluorescence staining for ERG in cell lines and flow cytometry analysis. The cancer cell lines used in this study were obtained from ATCC. VCaP cells were grown in DMEM/10%FCS+Penicillin/Streptomycin/L-Glutam ine. LNCaP, T2, Jurkat, KG-1 and KG-1a cells were

cultured in RPMI/10%FCS/Penicillin/ Streptomycin/L-Glutamine. On the day of the assay adherent cell lines (VCaP, LNCaP) were trypsinized to yield single cell suspensions while the non-adherent cells (T2, Jurkat, KG-1 and KG-1a) were utilized after a washing step. For each cell line, two aliquots of $5x10^5$ cells/tube were permeabilized with freshly prepared permeabilization buffer as directed by the manufacturer (eBioscience). One aliquot was stained with an IgG1 isotype control antibody (Invitrogen) and the second aliquot was stained with the anti-ERG antibody at a 1:20 dilution for 1 hour at 4°C. Then cells were washed and stained with FITC-conjugated rat anti-mouse IgG1 antibody (Clone A85-1, BD Pharmingen) for an additional 30 minutes at 4°C. Cells were then washed and analyzed by using a BD FACS Canto II flow cytometer. Data was collected on the total cell population and the analysis was performed by using FACS Diva software (version 5 .03) (Becton Dickinson).

Tissues and processing for Immunohistochemistry: Fixation and impregnation FVB/N and C57BL6 mice embryos from various stages of development were dissected from pregnant females, washed in ice-cold phosphate-buffered saline (PBS) and fixed in ice-cold 4% paraformaldehyde overnight, sequentially dehydration, embedding, and sectioning steps were performed according to established protocols.

Preparation for staining: Slides (with 5-mm sections) were warmed at 60°C for 15 minutes and immediately immersed in xylene for effective removal of paraffin and processed additionally twice in xylene for 5 min each, washed twice in 100% ethanol for 5 min each, and subsequently rehydrated in 95, 75% ethanol, and PBS for 5 min at room temperature.

Immunohistochemistry: Antigen retrieval was performed on these slides in Antigen unmasking solution (Vector Biolabs, Burlingame, CA) by using vegetable steamer for 45 minutes and the slides were allowed to cool to room temperature for about 30 min. Slides were washed twice in 1xPBS and treated with 3% H_2O_2 in ultrapure water for 15 minutes to quench the endogenous peroxidase activity. Immunodetection was performed using Mouse–to-mouse detection system kit according manufacturer's instructions (Millipore Inc. Billerica, MA). Briefly, nonspecific binding of the antibody was blocked by incubating the slides with pre-blocking solution for 10 minutes at RT, primary antibodies (200 pg/ml in 10% normal goat serum) at 4°C overnight or room temperature for 2 hours. Slides were washed twice in 1xPBS sites 5 min each, incubated with post-antibody blocking solution for 10 min. Slides were washed twice in 1xPBS

sites 5 min each and incubated with ready-to-use poly-HRP-Anti-Mouse/Rabbit IgG for 30 minutes, washed twice with 1xPBS for 5 minutes each. Color development was performed by using DAB as a substrate for peroxidase enzyme activity. The color reaction was stopped by washing/rinsing slides in tap water several times. Slides were counterstained with Hematoxylene for 2 minutes, rinsed in tap water for 5 mins, dehydrated sequentially in ascending concentration of alcohol, cleared in xylene and permanently mounted with Permont solution. The slides were scanned in Biomedical Instrumentation Center at Uniformed Services University of the Health Sciences using Olympus NanoZoomer Digital Pathology microscope at 40X magnification and digital images were taken from the scans.

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Conflict of Interest

The authors do not have any conflict of Interest.

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